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Expression of single-chain antibody fragments (scFv) specific for beet necrotic yellow vein virus coat protein or 25 kDa protein in *Escherichia coli* and *Nicotiana benthamiana*

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Abstract

The coding sequences for the variable regions of heavy and light chains of monoclonal antibodies (mAbs) to beet necrotic yellow vein virus (BNYVV) coat protein (cp) or the 25 kDa nonstructural protein (P25) were cloned into the pCOCK vector and expressed as single-chain antibody fragments (scFv) in *Escherichia coli*. For expression in higher plants the scFv were targeted either to the secretory pathway by including the sequences encoding the pectate lyase B (PelB) or the phytohemagglutinin (PHA) signal peptides in the vector constructs or they were targeted to the cytoplasm by omitting a signal peptide encoding sequence from the constructs. The scFv were detected mainly in plants in which the PHA signal peptide had been used for targeting demonstrating for the first time the usefulness of this peptide for enabling scFv expression in plants. The scFv were not secreted into the culture fluids of suspension cultures, but were retained in the cells. The amount of expression of scFv in the best expressing plants was at least as high as in bacterial culture supernatants. In a dot blot immunoassay, 0.4 ng BNYVV cp or 0.8 ng P25 were detected by the respective scFv either from *E. coli* or from plants. The majority of the 21 plants expressing cp specific scFv had near-normal growth whereas the three plants expressing P25-specific scFv grew poorly and did not form roots.

Introduction

Complete antibodies as well as single-chain antibody fragments (scFv) in which the variable domains of antibody heavy and light chains (V_H and V_L) are joined together by a linker peptide have recently been expressed in plants for inhibition of physiological processes [29] or the establishment of virus resistance [33, 36]. In antibody-producing B lymphocytes the immunoglobulin chains are targeted to the secretory pathway for correct folding and assembly by a specific immunoglobulin signal peptide. This signal peptide and signal peptides for other proteins in plants have been used to enable the production of either complete functional antibodies or scFv in plants [13, 10, 9, 33, 35]. The expression of functional scFv without a signal peptide in the cytoplasm of plant cells has also been described [33].

To determine the influence of signal peptides on the production efficiency and accumulation site of BNYVV specific scFv in transformed *Nicotiana benthamiana*, the scFv-encoding sequences were linked to the coding sequences for either the plant signal peptide for *Phaseolus vulgaris* phytohemagglutinin (PHA) [6], or for the bacterial signal peptide for pectate lyase B (PelB) [24]. The latter has been reported

The nucleotide sequence data reported will appear in the EMBL Nucleotide Sequence Database under the accession numbers Z70661 (BNYVV coat protein-specific scFv) and Z70662 (P25-specific scFv).

to have some activity in higher plants [3]. In addition, constructs for cytoplasmic scFv expression lacked the coding sequence for a signal peptide. *N. benthamiana* was chosen because it is more readily transformed than sugar-beet and can be infected by some isolates of BNYVV. The coding sequences for the scFv were derived from two hybridoma cell lines secreting monoclonal antibodies (mAbs) specific for BNYVV coat protein (cp) or the 25 kDa non-structural protein (P25), respectively. The cp specific mAb reacted with epitope 4b which is exposed along the entire length of the virus particles [5]. The P25 is the major protein encoded on BNYVV RNA 3 which is mainly responsible for the yield losses and the beard formation observed in BNYVV-infected sugar-beet [32, 21, 20]. Functionally active scFv were detected mainly in plants transformed with constructs containing the coding sequence for the PHA signal peptide, demonstrating for the first time the usefulness of this signal peptide for the expression of scFv in plants.

Materials and methods

Production of recombinant BNYVV cp and BNYVV P25

The BNYVV cp gene was cloned into the pET vector [31] for expression in *E. coli* strain BL21 DE3 (Novagen). The transformed bacteria were lysed in SDS PAGE sample buffer [22] and the proteins were separated by SDS PAGE. The denatured cp was electrophoretically eluted into dialysis tubing in 25 mM Tris, 192 mM glycine, 0.02% SDS at pH 8.2 from excised gel pieces [30]. Recombinant histidine-tagged BNYVV P25 was produced in *E. coli* strain BL21-DE3 and purified by means of Ni-NTA affinity chromatography [19].

PCR primers for cloning scFv encoding sequences

Primers for amplification of DNA encoding V_H and the heavy-chain constant domain (C_H1): **MOCG12For**, 5′-d(CTCAATTTTCTTGTCCACCTTGGTGC)-3′; **MOCG3For**, 5′-d(CTCGATTCTCTTGATCAACTCAGTCT)-3′; **VH1Back**, 5′-d(AGGGTSMARCTGCAGSAGTCWGG)-3′; S=C or G; M=A or C; R=A or G; W=A or T. Primers for the amplification of DNA encoding V_L and the constant domain (C_L): **CKFor**, 5′-d(CTCATTCCTGTTGAAGCTCTTGAC)-3′; **VK2Back**, 5′-d(GACATTGAGCTCACCCAGTCTCCA)-3′. Primers for cloning DNA encoding V_H: **VH1For2 LIAscI**, 5′-d(ACCGCCAGAGGCGCGCCCACCTGAACCGCCTCCACCTGAGGAGACGGTGACCGTGGTCCCTTGGCCCC)-3′; **VH1BackSfiI**, 5′-d(CATGCCATGACTCGCGGCCCAGCCGGCCATGGCCSAGGTSMARCTGCAGSAGTCWGG)-3′. Primers for cloning DNA encoding V_L: **JK2ForNot I**, 5′-d(GAGTCATTCTGCGGCCGCCCGTTTTATTTCCAGCTTGGTCCC)-3′; **VK2BackLiAscI**, 5′-d(GGTTCAGATGGGCGCGCCTCTGGCGGTGGCGGATCGGACATTGAGCTCACCCAGTCTCCA)-3′ [4, 27, 7]. Primers for cloning of DNA encoding scFv connected to the PelB or PHA signal peptides: **pelBBackBspHI**, 5′-d(GACAGTTCATCATGAAATACC)-3′; **VKForXbaI**, 5′-d(TATGCTCTAGATTCAACAGTCTATGCGGC)-3′; **PHABackNcoI**, 5′-d(ATCAGCCATGGCTTCCTCCAAGTTCTTCACTGTCCTCTTCCTTGTGCTTCTCACCCACGCAAACTCAAGCAACGATSAGGTCCAGCTGCAGSAGTCWGG)-3′.

Cloning of V_H and V_L encoding sequences

Poly(A)$^+$ RNA was isolated by means of the Pharmacia mRNA purification kit from murine monoclonal hybridoma cells secreting either BNYVV cp- or P25-specific mAbs. First strand cDNA was synthesized by means of M-MLV reverse transcriptase (Gibco-BRL). DNA fragments encoding the variable regions of the heavy or the light chains (V_H or V_L) and parts of their constant domains were obtained after PCR amplification using MOCG12For or MOCG3For and VH1Back or CKFor and VK2Back as primers. These PCR products served as templates for amplifying the V_H and V_L sequences in a second reaction using the primer pairs VH1For2LiAscI/VH1BackSfiI or JK2ForNotI/VK2BackLiAscI, respectively. The amplified DNA fragments were cloned sequentially into the bacterial expression vector pCOCK [7] and the recombinant plasmids were used to transform *E. coli* HB2151 [15]. The nucleotide sequences of scFv-encoding DNA fragments were determined by using the DNA-Sequenase kit Version 2.0 (United States Biochemical Corporation).

Expression of scFv in bacteria and analysis of their functionality by means of dot blot and western blotting analyses

Induction of the lac promoter for scFv production was performed by adding IPTG to a final concentration of

1 mM to transformed HB2151 cells grown at 37 °C to an OD_{600} of 0.6–0.8 in 2 × YT medium [30] supplemented with 0.05% glucose and 50 mg/l ampicillin. After the addition of IPTG the cultures were incubated at 28 °C for an additional 12–16 h. Bacteria were then centrifuged at 4500 × *g* for 15 min, the supernatants were freed from residual bacteria by filtration through a Schleicher & Schüll FP 030/3 membrane (pore size 0.2 μm) and were stored either at −20 °C or +4 °C. Periplasmic extracts were prepared according to Minski *et al.* [25] with the following modifications: Cells from a 30 ml culture were resuspended in cold (+4 °C) spheroplast buffer containing 0.5 M sucrose, 0.05 mM EDTA, 0.2 M Tris pH 8, and 120 μl of a 10 mg/ml lysozyme solution as well as 1060 μl of 0.5× cold spheroplast buffer were added. After incubation for 30 min on ice, the spheroplasts were pelleted by centrifugation at 12 000 × *g*, and the supernatant was used as periplasmic fraction. After western blotting scFv were detected in bacterial fractions by means of the c-myc peptide (Myc tag [8]) specific mAb 9E10 and anti-mouse IgG alkaline phosphatase-conjugated rabbit antibodies. For detecting the binding of the scFv to their respective antigens, recombinant BNYVV cp or P25 were immobilized on a membrane either in western blotting or a dot blot immunoassay. In the latter assay the antigens were dotted in several dilutions onto stripes of a Qiagen nylon membrane. After blocking their free protein-binding sites by incubation in 50 mM Tris, 150 mM NaCl, 0.05% Tween, 2% skimmed milk powder at pH 7.5 for 30 min, the stripes were treated for 1–2 h with the respective scFv preparations. After washing with the same buffer without milk powder the bound scFv were detected by means of the mAb 9E10 as described above.

Plant expression vectors and detection of functionally active scFv in plant extracts

The coding regions for either the BNYVV cp- or the P25-specific *scFv* together with the PelB signal peptide-encoding sequence were amplified from the respective pCOCK vector constructs by means of PCR using the primers pelBBackBspHI and VKForXbaI. One aliquot of each DNA sample was digested with *Nco*I to remove the signal peptide-encoding sequence (Fig. 1). The other aliquot was treated with *Bsp*HI to produce DNA fragments which still contain the PelB signal peptide-encoding sequence. A third type of scFv gene-containing DNA fragment which contained the coding sequence for the PHA signal peptide of *Phaseolus vulgaris* [6] was amplified from the respective pCOCK vector constructs by using the primers PHABackNcoI and VKForXbaI. These DNA samples were first digested with *Nco*I. All three types of DNA fragments for each of the two scFv were then digested with *Xba*I and integrated between the *Nco*I and *Xba*I restriction sites of either pRT103 [34] or pRT213, a pRT103-derived vector with an enhanced CaMV 35S promoter [28] and the Ω-leader region of TMV [11]. The various types of vector constructs were used to transform *E. coli* strains DH5α [12] and HB2151 [15]. The intactness of the scFv-encoding sequences connected either to the bacterial PelB or the plant PHA signal peptide-encoding sequences was tested after transforming *E. coli* HB2151 with the respective pRT vectors. The weak activity of the 35S promoter in *E. coli* [2] allowed a low level of scFv expression. The PelB and the PHA signal peptides mediated the scFv transport into the periplasmic space. The expression cassettes from the pRT vectors were then integrated into the binary vector pLX222 [23]. *Agrobacterium tumefaciens* LBA4404 Rif^r [14] was transformed as described [1] and used for transforming *N. benthamiana* by a modified leaf disk method [16].

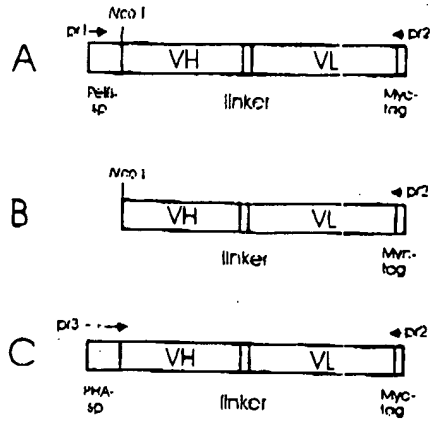

Figure 1. Gene constructs for the expression of the BNYVV cp- and P25-specific scFv in plants. The respective scFv encoding constructs were integrated into the CaMV 35S promoter expression cassettes of either pRT103 or pRT213, respectively. A and C: Constructs encoding the scFv together with the PelB (PelB-sp) or PHA (PHA-sp) signal peptides for targeting the scFv to the secretory pathway. B: Construct for the expression of the scFv in the cytoplasm. V_H and V_L: linker, Myc tag: DNA sequences encoding the variable regions of antibody heavy and light chains, the linker peptide and the Myc tag marker peptide, respectively; *Nco*I: cleavage site for *Nco*I; pr1–pr3: initiation sites of PCR primers (pr1 = PelBBackBspHI; pr2 = VKForXbaI; pr3 = PHABackNcoI).

Extracts from the regenerated kanamycin-tolerant plants were obtained by grinding 0.02–2 g of leaves with twice the amount of 50 mM Tris, 0.15 M NaCl, 0.25% Nonidet P40, 1.3% polyvinylpyrollidone, 0.001% Chymostatin, 0.05% Pefabloc (Boehringer) pH 7.5. These extracts were tested for neomycine phosphotransferase by means of ELISA (5Prime → 3Prime, Inc.) and their protein content was determined by means of the the Pierce BCA protein assay reagent. For checking the functionality of the scFv in plant extracts, 70 ng of recombinant cp or 200 ng of recombinant P25 in a volume of 0.7 µl each was dotted onto small Qiagen nylon membrane disks of 5 mm diameter. These disks were treated in the same way as the membrane stripes described above with the difference that all incubations were performed in the wells of a microtiter plate.

In order to obtain a rough estimate of the scFv concentration in plants, the color intensities obtained for plant extracts in the dot blot immunoassay with immobilized antigens were compared with those obtained with various dilutions of a denatured scFv preparation from bacteria which were dotted directly onto the membrane. This preparation had been obtained after elution of the scFv band from an SDS-polyacrylamide gel. Its protein content had been determined by means of the Pierce BCA protein assay reagent.

Establishment of suspension cultures

Calli were obtained from transgenic plants by incubating sterile leaf pieces for several weeks on solid MS medium supplemented with 200 mg/l Claforan, 50 mg/l kanamycin sulfate and 0.44 mg/l 2,4 dichlorophenoxyacetic acid (2,4 D). Loose callus tissue was transferred into liquid MS medium supplemented with 100 mg/l Claforan, 25 mg/l kanamycin sulfate and 0.44 mg/l 2,4-D and was incubated for 2–20 days on a rotary shaker at 25 °C. After settling for ca. 10 min a smaller fraction with the plant cells and a larger fraction consisting of the culture medium were readily separated. Each fraction was mixed with an equal volume of 50 mM Tris, 0.15 M NaCl, 0.25% Nonidet P40, 1.3% polyvinylpyrollidone, 0.001% Chymostatin, 0.05% Pefabloc (Boehringer) pH 7.5. The culture supernatant devoid of cells was concentrated by ultrafiltration to give the same volume as the cell fraction. The cell fraction was homogenized and an aliquot of this homogenate was centrifuged for 5 min at 13 000 × *g*.

Results

Expression of BNYVV cp- and P25-specific scFv in E. coli

Although starting with two mAb-secreting hybridoma cell lines each for BNYVV cp or for BNYVV P25, scFv that reacted were obtained only with one cell line for each of the two proteins. Even with these two cell lines only a few of the pCOCK-derived clones which were obtained with the respective V_H- and V_L-encoding DNA fragments enabled the expression of functionally active scFv of the expected size in *E. coli* strain HB2151 (Fig. 2A). The cp-specific scFv reached the highest concentration in the culture supernatants of the bacterial cultures, whereas the P25-specific scFv were found predominantly in the periplasmic fraction of the bacteria.

The coding sequences for the cp- and the P25-specific scFv consisted of 783 and 795 nucleotides, respectively (including the coding sequence for the Myc tag peptide, but without the coding sequence for the PelB signal peptide). The calculated molecular masses of 28.0 and 28.4 kDa for the translation products agreed well with those determined for the two scFv by means of SDS-PAGE and western blotting, i.e. 29 and 30 kDa, respectively (Fig. 2A). According to the classification of Kabat *et al.* [18] the coding sequences of the cp-specific V_H and V_L regions belong to subgroups III(C) and I, whereas those of the P25-specific V_H and V_L regions belong to groups II(B) and III, respectively.

In the dot blot immunoassay the two scFv detected their immobilized homologous antigens with almost the same sensitivity as the original mAb from which they were derived, i.e. 0.4 ng cp (Fig. 2B, lane a) and 0.8 ng P25 (data not shown). None of the two scFv cross-reacted with crude plant sap or other antigens in this type of test. In ELISA on plates precoated with polyclonal antibodies the cp-specific scFv also allowed the highly sensitive and specific detection of BNYVV particles; the P25-specific scFv, on the other hand, showed also some reactivity with sap from healthy plants.

Expression of BNYVV cp- and P25-specific scFv in N. benthamiana

Three different types of scFv-encoding DNA fragments were generated from the respective pCOCK constructs by means of PCR using specific primer sets

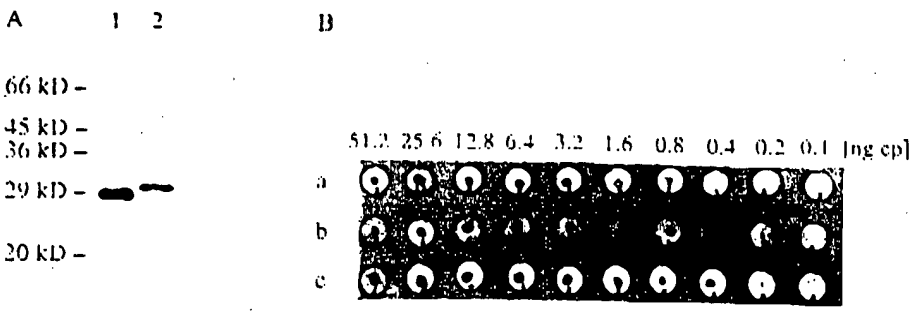

Figure 2. A. Detection of the cp- and the P25-specific scFv clones 1 and 2, respectively) expressed in *E. coli* in western blots by means of the Myc-tag marker peptide specific mAb 9E10 and alkaline phosphatase labelled rabbit anti mouse antibodies. B. Detection of BNYVV cp at various dilutions in a dot blot immunoassay on small membrane disks by means of (a) cp-specific scFv from an undiluted culture supernatant from transformed *E. coli*, (b) cp-specific scFv in an extract from a plant transformed with a construct containing the coding sequence for the PHA signal peptide (dilution 1:4), (c) the hybridoma culture supernatant secreting the cp specific mAb from which the scFv were derived (dilution 1:500). The lines on the disks are labels marking the antigen containing site of the disks during experimental procedures.

(Fig. 1). They either lacked a coding sequence for a signal peptide or they contained the coding sequences for the PelB signal peptide from bacteria [24] or the PHA signal peptide from plants [6]. Expression cassettes for scFv production in plants were obtained after the integration of these fragments between the CaMV 35S promoter and the CaMV 35S polyadenylation signal of pRT103 or between the enhanced 35S promoter with the tobacco mosaic virus Ω-leader region and the CaMV 35S polyadenylation signal of pRT213 (see Materials and methods).

BNYVV cp- and P25 specific scFv were detected mainly in plants which had been transformed with constructs containing the coding sequence for the PHA signal peptide (Table 1). A very weak expression (less than 0.001% of total protein) of functionally active cp-specific scFv was also recorded in three plants which had been transformed with constructs containing the coding sequence for the PelB signal peptide. No functionally active scFv were found in plants transformed with constructs lacking the coding sequence for a signal peptide.

Most of the plants expressing the cp-specific scFv showed a more or less normal growth, however, the three plants which expressed the P25-specific scFv grew only poorly and none of them formed roots. The 26 non-expressing plants showed a normal phenotype. No plants at all were regenerated from leaf disks treated with *A. tumefaciens* containing the expression constructs for the P25-specific scFv with the coding sequence for the PelB signal peptide, despite the fact that twice as many leaf disks were used for transformation than with the other constructs (Table 1).

The concentration of the cp-specific scFv in the four well expressing plants was estimated to amount to about 0.007–0.01% of the total protein (Table 1) and was at least as high as in the supernatants of the *E. coli* cultures described in the previous section. As little as 0.1 ng BNYVV cp were readily detected with extracts from such plants (Fig. 2B, lane b). After selfing of plants showing the highest scFv production, plants of the F2 generation were selected with an scFv content of roughly 0.05–0.1% of the total protein.

Cell suspension cultures were obtained from several plants transformed with constructs for the expression of cp-specific scFv with or without a coding sequence for a signal peptide. No functionally active scFv were detected in any of the culture fluids 48 hours after the transfer of suspension cultures to fresh medium; functionally active scFv were detected, however, in the supernatants of cell homogenates of suspension cultures derived from well expressing plants in which the PHA signal peptide had been used for targeting. Very small amounts (weakest positive signal in the dot blot immunoassay) of functionally active scFv were also detected in the culture fluids of suspension cultures 2 to 3 weeks old derived from plants in which a signal peptide-encoding sequence was omitted from the vector constructs in order to enable a cytoplasmic scFv expression.

Discussion

We have demonstrated that scFv specific for BNYVV cp or 25K non-structural protein can be expressed in bacteria as well as in *N. benthamiana*. The highest

Table 1. Detection of the expression of BNYVV cp and P25-specific scFv in *Nicotiana benthamiana* transformed with various gene constructs.

Specificity scFv	pICI vector	Coding sequence for signal peptide in the expression cassette	Number of leaf disks treated with transformed *Agrobacterium*	Total number of kanamycin resistant plants regenerated	Number of plants with strong* scFv production	medium* or low * scFv produciton	no detectable scFv production+
BNYVV cp	103	none	60	39	none	none	39
	213	none	60	10	none	none	10
	103	PelB	60	5	none	3	2
	213	PelB	60	3	none	none	3
	103	PHA	60	25	none	7	18
	213	PHA	60	13	4	7	2
BNYVV P25	103	none	30	none	none	none	none
	213	none	30	13	none	none	13
	103	PelB	60	none	none	none	none
	213	PelB	60	none	none	none	none
	103	PHA	30	21	1	none	20
	213	PHA	30	8	2	none	6

* The scFv concentrations were roughly estimated at [illegible] between 0.002–0.01% in the plants with a 'strong scFv production' and to ≤ 0.0002% of total protein in the plants with 'medium or low scFv production'.
+ Positive results in the neomycin phosphotransferase test indicated that these plants were transformed.

detectable amounts of scFv were found in plants when the scFv were targeted to the secretory pathway by means of the PHA signal peptide. Its usefulness for expressing scFv in plants was demonstrated here for the first time. Our experiments with suspension cultures indicated that the cp specific scFv were retained in the cells (presumably the endoplasmic reticulum (ER)) rather than excreted. This is in contrast to observations of Hunt *et al.* [17] who found that the PHA signal peptide directed the secretion of pea seed albumin into the culture fluid. Whether a protein is retained in a plant cell may depend on the signal peptide and perhaps on the protein to be targeted or the plant species. A secretion of complete antibodies from plant cells was observed when the immunoglobulin signal peptide was used for targeting [36, 35]. If the BNYVV cp-specific scFv, which presumably has accumulated in the ER, will interfere with the activities of the virus in infected plants is yet to be determined.

Various scFv lacking a signal peptide apparently also differ in their suitability for cytoplasmic expression. Tavladoraki *et al.* [33] observed a cytoplasmic expression with scFv specific for artichoke mottled crinkle virus (AMCV) that lacked a signal peptide, whereas we failed to do so with scFv specific for BNYVV cp and P25. We cannot exclude the possibility that some of our plants actually produced scFv that in contrast to the AMCV-specific scFv were highly sensitive to proteolytic digestion or the reducing environment in the cytoplasm. Very low amounts of functionally active scFv were detected in culture supernatants of supension cultures from plants in which the scFv had been targeted to the cytoplasm by omitting a signal peptide encoding a sequence from the vector constructs. These scFv possibly had correctly folded immediately after cell lysis in the less reducing environment of the culture fluid. In approaches aimed to induce virus resistance in plants a cytoplasmic scFv expression may be especially desirable, because it would enable an interaction between the scFv and the targeted viral proteins in the compartment where the latter are produced.

scFv are not only interesting as potential inhibitors of virus multiplication in plants, but also as potential diagnostic reagents for detecting virus infections. When the genetic information for the scFv is obtained from large combinatorial libaries [26] the use of animals as producers of antibodies may eventually become unnecessary. Our results show that BNYVV-specific scFv can be produced as efficiently in plants as in bacteria.

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